

Plant phosphomevalonate kinases

5 The invention relates to nucleic acids which encode plant polypeptides with the biological activity of phosphomevalonate kinases, to the polypeptides encoded by them and their use as targets for herbicides and their use for identifying novel, herbicidally active compounds, and to methods for finding modulators of these polypeptides.

10 Unwanted plant growth can be prevented by using herbicides. The demands made on herbicides with regard to their efficacy, costs and environmental compatibility have been steadily increasing. There exists therefore a demand for new substances which can be developed into potent new herbicides. In general, it is usual to search for such new lead structures in greenhouse tests. However, such tests are laborious and
15 expensive. Accordingly, the number of substances which can be tested in the greenhouse is limited.

Advantageous targets for herbicides are searched for in essential biosynthetic pathways. Thus, the biosynthesis of isoprenoids in plants leads, inter alia, to the
20 synthesis of carotenoids and of the side chains of plastoquinone and of chlorophyll. These products are essential for the photosynthetic growth of plants. The inhibition of one step in this biosynthetic pathway leads to the termination of a plant's growth. Moreover, plant hormones such as gibberellic acid, abscisic acid and brassinosteroids and membrane components (phytosterols), which are also essential for the plant's
25 growth, are formed from isoprenoids.

Isopentyl diphosphate (IPP) is the branching point from which the widest range of isoprenoids are formed. The production of IPP is therefore a critical point in plant metabolism. In plants, IPP is produced via two different metabolic pathways in
30 different compartments. In the endoplasmic reticulum (ER) and in the cytosol, IPP synthesis proceeds via the classic acetate/mevalonate metabolic pathway as it also

proceeds in the animal organism. In contrast, IPP is synthesized in chloroplasts via the alternative glyceraldehyde phosphate/pyruvate metabolic pathway. Both metabolic pathways are essential since various isoprenoid metabolites are formed in the different compartments. Moreover, the degree to which the two metabolic pathways are autonomous or to which an exchange of metabolites takes place between the compartments has not been elucidated as yet (Heintze et al., 1990, Kleinig, 1989).

Clomazone is a known herbicidal compound which reduces the carotenoid and chlorophyll content in the leaf. For a long time it has been assumed that clomazone acts via the inhibition of the isoprenoid metabolic pathway. Norman et al. (1990) had demonstrated that the site of action would have to be between mevalonate and geranylgeranyl pyrophosphate. This would establish one of the interposed five enzymes, one of which is phosphomevalonate kinase, as the site of action. Somewhat more recent works by Weimer et al. (1992) and Rodney Croteau (1992) suggest, however, that the site of action of clomazone would be found elsewhere.

Within the context of the present invention, an *Arabidopsis thaliana* cv. Columbia cDNA has been isolated with homology to phosphomevalonate kinase, hereinbelow abbreviated to PMVK, from *Saccharomyces cerevisiae* (Fig. 1). It was possible to induce this gene in *Arabidopsis thaliana* cv. Columbia by treatment with the herbicide chlorsulfuron (10 g/ha).

The homology between the *Saccharomyces cerevisiae* PMVK (= ERG8) and the cDNA isolated from *A. thaliana* amounts to 44% similarity or 35% identity (see Fig. 1, Bestfit with Wisconsin Package Version 10.1). This corresponds for example to the homology between the *Saccharomyces cerevisiae* mevalonate kinase and the *Arabidopsis thaliana* mevalonate kinase with a similarity of 45% and an identity of 35%. In the case of *Arabidopsis thaliana* mevalonate kinase the function can be confirmed by complementation of the corresponding mutant from *Saccharomyces cerevisiae*. Moreover, the cDNA isolated within the context of the present invention shows 69%

identity with a partial PMVK sequence from *Pinus radiata* in accordance with SEQ ID NO:5, which is of interest for modifying the isoprenoid content, isoprenoid composition and isoprenoid metabolism of plants (WO 00/36 081). Further partial cDNAs from plants (*Medicago trunculata*, Accession Number AA660847, see SEQ ID NO:3 and *Gossypium hirsutum*, Accession Number AI727861, see SEQ ID NO:4) have been isolated as putative PMVKs. Various *Arabidopsis* spp. sequences (ESTs and genomic sequences) which correspond to the PMVK sequence isolated herein or to parts thereof can be found in databases from various sequencing projects, however, no information is given on the function or importance of these sequences or sequence fragments.

For the first time, the present invention now provides the complete cDNA sequence of a plant phosphomevalonate kinase and describes its use, or the use of the polypeptides encoded thereby, for identifying new herbicidal active compounds.

Subject-matter of the present invention are therefore nucleic acids which encode complete plant phosphomevalonate kinases, with the exception of the partial nucleic acid sequences from *Medicago trunculata* in accordance with SEQ ID NO:3, *Gossypium hirsutum* in accordance with SEQ ID NO:4 and *Pinus radiata* in accordance with SEQ ID NO:5.

Subject-matter of the present invention are, in particular, nucleic acids which encode the *Arabidopsis thaliana* phosphomevalonate kinase.

Subject-matter of the present invention are very particularly nucleic acids which encode the *Arabidopsis thaliana* phosphomevalonate kinase and are described under SEQ ID NO:1 and/or encode a polypeptide in accordance with SEQ ID NO:2 or fragments thereof.

The nucleic acids according to the invention are, in particular, single-stranded or double-stranded deoxyribonucleic acids (DNA) or ribonucleic acids (RNA).

Preferred embodiments are fragments of the genomic DNA, which may contain introns, and cDNAs.

The nucleic acids according to the invention are preferably DNA fragments which correspond to the cDNA of Arabidopsis plants.

The nucleic acids according to the invention especially preferably encompass a sequence selected from amongst

- 10 a) the sequence in accordance with SEQ ID NO: 1,
- b) sequences which encode a polypeptide which encompasses the amino acid sequence in accordance with SEQ ID NO: 2,
- 15 c) part sequences of the sequences defined under a) or b) which have a length of at least 14 base pairs,
- d) sequences which hybridize with the sequences defined under a) or b) at a hybridization temperature of 35-52°C,
- 20 e) sequences which have at least 70% identity, preferably 85% identity, especially preferably 90% identity, very especially preferably 95% identity, with the sequences defined under a),
- 25 f) sequences which have at least 70% identity, preferably 80% identity, especially preferably 90% identity, very especially preferably 95% identity, with the sequences defined under b),
- 30 g) sequences which are complementary to the sequences defined under a) or b), and

- h) sequences which, owing to the degeneracy of the genetic code, encode the same amino acid sequence as the sequences defined under a) to f).

5 A cDNA molecule with the sequence in accordance with SEQ ID NO: 1 constitutes a very especially preferred embodiment of the nucleic acids according to the invention.

10 The term "complete" phosphomevalonate kinase as used in the present context describes the phosphomevalonate kinase which is encoded by a complete coding region of a transcription unit starting with the ATG start codon and encompassing all information-bearing exon regions of the gene present in the organism of origin and encoding phosphomevalonate kinase, and the signals required for correct termination of transcription.

15 The term "gene" as used in the present context refers to a section from the genome of a cell which section is responsible for the synthesis of a polypeptide chain.

20 The term "to hybridize" as used in the present context describes the process in which a single-stranded nucleic acid molecule undergoes base pairing with a complementary strand. In this manner, for example, DNA fragments which encode phosphomevalonate kinases which exhibit the same or similar properties as the kinase with the amino acid sequence in accordance with SEQ ID NO: 2 can be isolated from plants other than Arabidopsis, starting from the sequence information disclosed herein.

25 The term "cDNA" as used in the present context refers to the single- or double-stranded copy of an RNA molecule and, being a copy of biologically active mRNA, is free from introns, i.e. all coding regions of a gene are present in connected form.

30 Hybridization conditions are calculated by approximation using the following formula:

Melting temperature $T_m = 81.5^{\circ}\text{C} + 16.6 \log[c(\text{Na}^+)] + 0.41(\%G + C) - 500/n$
(Lottspeich and Zorbas, 1998).

In this formula, c is the concentration and n the length of the hybridizing sequence
5 segment in base pairs. For a sequence >100 bp, the term $500/n$ does not apply. At the
highest stringency, washing is effected at a temperature of $5\text{-}15^{\circ}\text{C}$ below T_m and an
ionic strength of 15 mM Na^+ (corresponds to $0.1 \times \text{SSC}$). If an RNA probe is used for
hybridization, the melting point is $10\text{-}15^{\circ}\text{C}$ higher.

10 Preferred hybridization conditions are indicated hereinbelow:

Hybridization solution: DIG Easy Hyb (Roche)

Hybridization temperature: $35\text{-}52^{\circ}\text{C}$, preferably 42°C (DNA-DNA), or 50°C
(DNA-RNA).

- 15 1. Wash step: $2 \times \text{SSC}$, twice 5 minutes at room temperature;
2. Wash step: twice 15 minutes in $1 \times \text{SSC}$, at 50°C ; preferably $0.5 \times \text{SSC}$, at 65°C ;
especially preferably $0.2 \times \text{SSC}$, at 65°C .

20 The degree of identity of the nucleic acids is preferably determined with the aid of
the program NCBI BLASTN Version 2.0.14. (Altschul et al., 1997).

Subject-matter of the present invention are furthermore DNA constructs which
encompass a nucleic acid according to the invention and a homologous or
heterologous promoter.

25 The term "homologous promoter" as used in the present context refers to a promoter
which controls the expression of the gene in question in the organism of origin.

30 The term "heterologous promoter" as used in the present context refers to a promoter
which has properties other than the promoter which controls the expression of the
gene in question in the organism of origin.

5 The choice of heterologous promoters depends on whether pro- or eukaryotic cells or cell-free systems are used for expression. Examples of heterologous promoters are the cauliflower mosaic virus 35S promoter for plant cells, the alcohol dehydrogenase promoter for yeast cells, and the T3-, T7- or SP6 promoters for prokaryotic cells or cell-free systems.

10 Subject-matter of the present invention are furthermore vectors comprising a nucleic acid according to the invention, a regulatory region according to the invention or a DNA construct according to the invention. Vectors which can be used are all the phages, plasmids, phagemids, phasmids, cosmids, YACs, BACs, artificial chromosomes or particles which are suitable for particle bombardment that are used in molecular biology laboratories.

15 Preferred vectors are pBIN (Bevan, 1984) and its derivatives for plant cells, pFL61 (Minet et al., 1992) or, for example, the p4XXprom. series of vectors (Mumberg et al.) for yeast cells, pSPORT vectors (Life Technologies) for bacterial cells, lambdaZAP (Stratagene) for phages or Gateway vectors (Life Technologies) for various expression systems in bacterial cells or in baculovirus.

20 Subject-matter of the present invention are also host cells comprising a nucleic acid according to the invention, a DNA construct according to the invention or a vector according to the invention.

25 The term "host cell" as used in the present context refers to cells which do not naturally comprise the nucleic acids according to the invention.

30 Suitable host cells are not only prokaryotic cells, preferably *E. coli*, but also eukaryotic cells such as cells of *Saccharomyces cerevisiae*, *Pichia pastoris*, insects, plants, frog oocytes and mammalian cell lines.

Subject-matter of the present invention are furthermore polypeptides with the biological activity of phosphomevalonate kinases which are encoded by the nucleic acids according to the invention.

5 The term "polypeptides" as used in the present context relates not only to short amino acid chains which are usually termed peptides, oligopeptides or oligomers, but also to longer amino acid chains which are usually termed proteins. It encompasses amino acid chains which can be modified either by natural processes such as posttranslational processing or by chemical prior-art methods. Such modifications
10 can occur at various positions and repeatedly in a polypeptide, such as, for example, at the peptide backbone, at the amino acid side chain, at the amino and/or at the carboxy terminus. They encompass for example acetylations, acylations, ADP ribosylations, amidations, covalent linkages to flavins, haeme moieties, nucleotides or nucleotide derivatives, lipids or lipid derivatives or phosphatidylinositol, cyclizations, the formation of disulphide bridges, demethylations, the formation of
15 cystine, formylations, gamma-carboxylations, glycosylations, hydroxylations iodinations, methylations, myristoylations, oxidations, proteolytic processings, phosphorylations, selenoylations and tRNA-mediated additions of amino acids.

20 The polypeptides according to the invention may exist in the form of "mature" proteins or as parts of larger proteins, for example as fusion proteins. They may furthermore have secretion or leader sequences, pro-sequences, sequences which allow simple purification, such as repeated histidine residues, or additional stabilizing amino acids.

25 The polypeptides according to the invention, in particular the polypeptide in accordance with SEQ ID NO:2, need not constitute complete plant phosphomevalonate kinases, but may also just be fragments of these as long as they retain at least the biological activity of the complete plant phosphomevalonate
30 kinase. Polypeptides which exert the same type of biological activity as a phosphomevalonate kinase with an amino acid sequence in accordance with SEQ ID

NO:2 are still considered to be in accordance with the invention. The polypeptides according to the invention in this case need not be able to be derived from Arabidopsis phosphomevalonate kinases. Polypeptides which correspond to phosphomevalonate kinases of, for example, the plants stated hereinbelow, or to fragments thereof, and which are still capable of exerting the biological activity thereof are still considered to be in accordance with the invention: tobacco, maize, wheat, barley, oats, rice, rye, tomatoes, legumes, potato plants, *Lactuca sativa*, other Brassicaceae, woody species, *Physcomitrella patens*.

Compared with the corresponding region of naturally occurring phosphomevalonate kinases, the polypeptides according to the invention may exhibit deletions or amino acid substitutions as long as they still exert at least the biological activity of the complete kinase. Conservative substitutions are preferred. Such conservative substitutions encompass variations, where one amino acid is replaced by another amino acid from among the following group:

1. Small aliphatic residues, nonpolar residues or residues of low polarity: Ala, Ser, Thr, Pro and Gly;
2. Polar, negatively charged residues and their amides: Asp, Asn, Glu and Gln;
3. Polar, positively charged residues: His, Arg and Lys;
4. Large aliphatic nonpolar residues: Met, Leu, Ile, Val und Cys; and
5. Aromatic residues: Phe, Tyr and Trp.

Preferred conservative substitutions can be seen from the following list:

Original residue	Substitution
Ala	Gly, Ser
Arg	Lys
Asn	Gln, His
Asp	Glu
Cys	Ser
Gln	Asn
Glu	Asp
Gly	Ala, Pro
His	Asn, Gln
Ile	Leu, Val
Leu	Ile, Val
Lys	Arg, Gln, Glu
Met	Leu, Tyr, Ile
Phe	Met, Leu, Tyr
Ser	Thr
Thr	Ser
Trp	Tyr
Tyr	Trp, Phe
Val	Ile, Leu

Subject-matter of the present invention are thus also polypeptides which exert at least the biochemical reaction of the formation of 5-pyrophosphomevalonate from 5-phosphomevalonate, such as phosphomevalonate kinase, and which encompass an amino acid sequence which exhibits at least 60% identity, preferably 80% identity, especially preferably 90% identity, very especially preferably 97-99% identity, with the sequence in accordance with SEQ ID NO: 2 over its entire length.

The degree of identity of the amino acid sequences is preferably determined with the aid of the program BLASTP + BEAUTY Version 2.0.14. (Altschul et al., 1997).

A preferred embodiment of the polypeptides according to the invention is the phosphomevalonate kinase (PMVK) with the amino acid sequence in accordance with SEQ ID NO: 2.

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The PMVK amino acid sequence has a potential ATP binding site in the region of amino acids 177 to 186, which is typical of kinases.

10

The term "biological activity of a phosphomevalonate kinase" as used in the present context refers to the ability to convert 5-phosphomevalonate into 5-pyrophosphomevalonate with consumption of ATP and formation of ADP.

15

The nucleic acids according to the invention can be prepared in the customary manner. For example, the nucleic acid molecules can be synthesized chemically in their entirety. It is also possible chemically to synthesize short sections of the nucleic acids according to the invention, and such oligonucleotides can be radiolabelled or labelled with a fluorescent dye. The labelled oligonucleotides can also be used for screening cDNA libraries generated starting from plant mRNA. Clones which hybridize with the labelled oligonucleotides are selected for isolating the DNA fragments in question. After characterization of the DNA which has been isolated, the nucleic acids according to the invention are obtained in a simple manner.

20

Alternatively, the nucleic acids according to the invention can be generated by means of PCR methods using chemically synthesized oligonucleotides.

25

The term "oligonucleotide(s)" as used in the present context denotes DNA molecules composed of 10 to 50 nucleotides, preferably 15 to 30 nucleotides. They are synthesized chemically and can be used as probes.

30

Subject-matter of the invention are also polypeptides with phosphomevalonate kinase activity which are encoded by an abovementioned DNA.

The skilled worker knows that the polypeptides of the present invention can be obtained by various routes, for example by chemical methods such as the solid-phase method. The use of recombinant methods is recommended for obtaining larger
5 amounts of protein. Expression of a cloned phosphomevalonate kinase gene or fragments thereof can be effected in a series of suitable host cells with which the skilled worker is familiar. To this end, a phosphomevalonate kinase gene is introduced into a host cell with the aid of known methods.

10 The integration of the cloned phosphomevalonate kinase gene into the chromosome of the host cell is within the scope of the present invention. Preferably, the gene or fragments thereof are introduced into a plasmid and the coding regions of the phosphomevalonate kinase gene or fragments thereof are linked functionally with a constitutive or inducible promoter.

15 The basic steps for generating the recombinant phosphomevalonate kinase are:

1. Obtaining a natural, synthetic or semisynthetic DNA which encodes phosphomevalonate kinase.
- 20 2. Introduction of this DNA into an expression vector which is suitable for expressing phosphomevalonate kinase, either alone or as fusion protein.
3. Transformation of a suitable, preferably prokaryotic, host cell with this
25 expression vector.
4. Growing this transformed host cell in a manner which is suitable for expressing phosphomevalonate kinase.
- 30 5. Cell harvest and purification of phosphomevalonate kinase by suitable known methods.

5 The coding region of phosphomevalonate kinase can be expressed in *E. coli* by the customary methods. Suitable expression systems for *E. coli* are commercially available, for example the expression vectors of the pET series, for example pET3a, pET23a, pET28a with His tag or pET32a with His tag for simple purification and thioredoxin fusion for increasing the solubility of the enzyme expressed, and pGEX with glutathione synthetase fusion, and the pSPORT vectors. The expression vectors are transformed into λ DE3 lysogenic *E. coli* strains, for example BL21(DE3), HMS 10 174(DE3) or AD494(DE3). After the growth of the cells under standard conditions which are familiar to the skilled worker, expression is induced by means of IPTG. After induction, the cells are incubated for 3 to 24 at temperatures from 18 to 37°C. The cells are disrupted by sonification in breaking buffer (10 to 200 mM sodium phosphate, 100 to 500 mM NaCl, pH 5 to 8). The protein expressed can be purified by chromatographic methods, in the case of protein expressed with His tag by 15 chromatography on an Ni-NTA column.

Expression of the protein in commercially available yeast strains (for example *Pichia pastoris*) or in insect cell cultures (for example Sf9 cells) constitutes another advantageous approach.

20 Alternatively, the proteins may also be expressed in plants.

Subject-matter of the present invention are also methods for finding chemical compounds which bind to the polypeptides according to the invention and modify 25 their properties. Owing to the many functions of the terpenoids which make necessary the formation of the precursor isopentyl diphosphate and thus the function of the phosphomevalonate kinase according to the invention, modulators which affect the activity of the enzyme constitute new growth-regulatory or herbicidally active compounds. Modulators may take the form of agonists or antagonists, or activators or 30 inhibitors.

Subject-matter of the present invention is therefore in particular also the use of plant phosphomevalonate kinases as targets for herbicides and their use in methods for finding modulators of this polypeptide. In such methods, the phosphomevalonate kinases can be employed directly, in extracts or in purified form, or they can be formed indirectly via expression of the DNA encoding them.

Subject-matter of the present invention is therefore also the use of nucleic acids encoding plant PMVK, DNA constructs comprising them, host cells comprising them, or of antibodies binding to PMVK for finding PMVK modulators.

The term "agonist" as used in the present context refers to a molecule which accelerates or increases the activity of phosphomevalonate kinase.

The term "antagonist" as used in the present context refers to a molecule which slows down or prevents the activity of phosphomevalonate kinase.

The term "modulator" as used in the present context constitutes the generic term for agonist or antagonist. Modulators can be small organochemical molecules, peptides or antibodies which bind to the polypeptides according to the invention. Other modulators may be small organochemical molecules, peptides or antibodies which bind to a molecule which, in turn, binds to the polypeptides according to the invention, thus affecting their biological activity. Modulators may constitute natural substrates and ligands or their structural or functional mimetics. The term "modulator", however, does not encompass the natural substrates and ATP.

The modulators are preferably small organochemical compounds.

The binding of the modulators to the phosphomevalonate kinases according to the invention can alter the cellular processes in a manner which leads to the death of the plants treated therewith.

Subject-matter of the present invention are therefore also modulators, preferably inhibitors of the enzymatic activity of plant phosphomevalonate kinases, which were found with the aid of one of the processes described hereinbelow for identifying modulators of the phosphomevalonate kinase protein or a polypeptide which is homologous therewith.

Subject-matter of the invention is additionally the use of modulators of phosphomevalonate kinase as herbicides.

The present invention furthermore encompasses methods for finding chemical compounds which modify the expression of the polypeptides according to the invention. Such "expression modulators" too may constitute new growth-regulatory or herbicidal active compounds. Expression modulators can be small organochemical molecules, peptides or antibodies which bind to the regulatory regions of the nucleic acids encoding the polypeptides according to the invention. Furthermore, expression modulators can be small organochemical molecules, peptides or antibodies which bind to a molecule which, in turn, binds to regulatory regions of the nucleic acids encoding the polypeptides according to the invention, thus affecting their expression. Expression modulators may also be antisense molecules.

The present invention therefore also extends to the use of modulators of the polypeptides according to the invention or of expression modulators as plant growth regulators or herbicides.

Subject-matter of the present invention are also expression modulators of phosphomevalonate kinases which are formed with the aid of an above-described method of identifying expression modulators of the phosphomevalonate kinase protein or a polypeptide which is homologous thereto.

Subject-matter of the invention is also the use of expression modulators as herbicides.

Other methods according to the invention include high-throughput screening (HTS). Both host cells and cell-free preparations comprising the nucleic acids according to the invention and/or the polypeptides according to the invention may be used for this purpose.

In order to find modulators of the polypeptides according to the invention, a synthetic reaction mix (for example products of *in vitro* transcription) or a cellular component, such as a crude cell extract, or any other preparation comprising the polypeptide according to the invention can be incubated together with a labelled substrate or ligand of the polypeptides in the presence and absence of a candidate molecule, which may be an agonist or antagonist. The ability of the candidate molecule to increase or inhibit the activity of the polypeptide according to the invention can be seen from an increased or reduced binding of the labelled ligand or an increased or reduced conversion rate of the labelled substrate. Molecules which bind well and lead to an increased activity of the polypeptides according to the invention are agonists. Molecules which bind well but do not trigger the biological activity of the polypeptides according to the invention are probably good antagonists.

The detection of the biological activity of the polypeptides according to the invention can be improved by what is known as a reporter system. Reporter systems in this regard encompass, but are not limited to, colorimetrically labelled substrates which are converted into a product, or a reporter gene which responds to changes in the activity or the expression of the polypeptides according to the invention, or other known binding tests.

Modulators of the polypeptide according to the invention can also be found via enzymatic tests. The change in the enzyme activity by suitable modulators can either be measured directly or indirectly in a coupled enzyme test. The measurement can be carried out for example via the change in absorption owing to the decrease or increase of an optically active compound.

10

A further example of a method by means of which modulators of the polypeptides according to the invention can be found is a displacement test, in which the polypeptides according to the invention and a potential modulator are combined under conditions suitable for this test with a molecule which is known to bind to the polypeptides according to the invention, such as a natural substrate or ligand or a substrate or ligand mimetic. The polypeptides according to the invention themselves can be labelled, for example radiolabelled or colorimetrically labelled, so that the number of polypeptides which are bound to a ligand or which have undergone conversion can be determined accurately. In this manner, the efficacy of an agonist or antagonist can be assessed.

Example 1

Isolation of the nucleic acid encoding *A. thaliana* PMVK

- 5 A 370 bp PMVK fragment was repeatedly isolated from leaf material of *Arabidopsis thaliana* cv. Columbia plants with the aid of the suppression subtractive hybridization method (Diatchenko et al., 1996).

10 Suppression subtractive hybridization constitutes a method of isolating differentially expressed genes. The two samples to be compared were on the one hand *Arabidopsis* plants which had been harvested 24 hours after treatment with a herbicide (chlorsulfuron, 10 g/ha) and, on the other hand, *Arabidopsis* plants which had been harvested 24 hours after a control treatment. The 370 bp PMVK fragment was isolated from the chlorsulfuron-treated plants in which PMVK transcription may have been induced by the treatment.

15 The fragment obtained was cloned into vector pTAdv (Clontech) and transformed into *E. coli* strain TOP10F'. The PMVK fragment was furthermore used as probe for virtual Northern (Clontech) blots and employed as probe for isolating the complete PMVK cDNA.

20

Isolation of the complete PMVK cDNA sequence

- 25 An *Arabidopsis* cDNA library by Life Technologies in the plasmid vector pSPORT was screened with the aid of Clontech's Cloncapture kit following the manufacturer's instructions. However, as opposed to the manufacturer's instructions, Biotin labelling of the PMVK fragment employed as probe was not carried out by means of PCR, but with the aid of Boehringer's Biotin High Prime kit.

The PMVK-enriched plasmid DNA was transformed into *E. coli* cells and plated out overnight. The resulting colonies were analysed by colony PCR with PMVK-gene-specific primers, and positive colonies were identified.

Cultures from the positive colonies were grown by methods known to the skilled worker, and the plasmid DNA was isolated and the DNA was subsequently sequenced.

Example 2

To verify differential PMVK expression in the response to chlorsulfuron, so-called virtual Northern blot analyses were carried out.

In a virtual Northern blot, cDNA is prepared from total RNA with Clontech's SMART method (see manufacturer's instructions) and amplified by PCR. A low enough number of PCR cycles is employed so that the amplification is still within the linear range of the PCR. In the present case, an optimum between 15 and 18 cycles emerged. The SMART cDNA is separated on an agarose gel by methods known to the skilled worker, transferred to a nylon membrane and hybridized with a DIG-labelled probe. This method permits study of the expression even of genes with a low expression level.

The result showed a low degree of induction of PMVK expression by chlorsulfuron.

Example 3

A potential assay system for identifying modulators of phosphomevalonate kinase is based on the ADP detection of the coupled pyruvate kinase/lactate dehydrogenase assay.

Phosphoenol pyruvate is converted to pyruvate by pyruvate kinase, and pyruvate is then subsequently converted to lactate by lactate dehydrogenase with consumption of NADH. The consumption of NADH can be monitored by the decreasing absorption at 340 nm.

5

In the reaction of PMVK, ADP is formed, which can be detected in the assays described. The effect of PMVK modulators on this reaction can thus also be determined with reference to an increase or decrease in the ADP content.

10

Figures and sequence listing

Figure 1

15

Determination of the homology between the *A. thaliana* phosphomevalonate kinase according to the invention in accordance with SEQ ID NO:2 and the known *S. cerevisiae* phosphomevalonate kinase (BESTFIT) by means of Bestfit (Wisconsin Package Version 10.1 (GCG)). The similarity is 44% and the identity 35%.

SEQ ID NO:1

20

Nucleic acid sequence encoding *A. thaliana* phosphomevalonate kinase.

SEQ ID NO:2

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Amino acid sequence of the *A. thaliana* phosphomevalonate kinase.

SEQ ID NO:3

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Nucleic acid fragment from *Medicago trunculata* (putative PMVK) of Accession Number AA 660847.

SEQ ID NO:5

Nucleic acid fragment from *Pinus radiata* (encoding PMVK in accordance with WO 00/36081).

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